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## REMARKS

Claims 1-13 and 15-98 constitute the pending claims in the present application. Claims 1-12, 24-27, 34-38, 40, 41, 44, 47-49, and 52 are withdrawn as being directed to a non-elected invention. Applicants cancel, without prejudice, claims 1-12, 38, 40-44, and 47-49. Applicants submit, however, that claims 25-27, 34-37, 52, and 53-86 are properly dependent on elected independent claims and should be considered together upon determining that such independent claims are allowable, pursuant to MPEP 809.02(c). Applicants add new claims 96-119.

Support for the subject matter of these claims is found throughout the specification. In particular, support for a PYY agonist or a PYY, and biologically active fragments thereof, having at least one of the recited functions (see, *e.g.*, claims 13, 21, 23, etc.) can be found at least at page 4, lines 34-37, where it is described that compounds of the invention, "PYY Therapeutic," comprise "a PYY peptide or PYY agonist of (*e.g.*, which mimics or enhances) PYY activity;" and at least at page 21, lines 34-36 where exemplary PYY functions are described. Support for the amendment to claim 94 and new claims 96-119 directed to methods using PYY agonists having at least 70, 80, 85, 90 % identity to SEQ ID NO:3 and having at least one of the recited functions can be found at least at page 6, lines 4-6, as amended; and page 4, lines 34-37 and page 21, lines 34-36, as previously described.

Applicants have also amended the specification. Reference to SEQ ID NO:2 in the paragraph beginning on page 6, line 1, of the specification has been amended to SEQ ID NO:3. Support for the amendment can be found at least at page 21, lines 32-33; page 22, lines 21-22; and the state of the art, where one of ordinary skill in the art would understand that PYY is a 36 amino acid polypeptide that begins and ends with a tyrosine, hence the name PYY, peptide tyrosine tyrosine, as shown in SEQ ID NO:3. No new matter has been entered.

Applicants thank the Examiner and her Supervisor for courtesies extended during a telephonic interview conducted January 9, 2004. Applicants respectfully request reconsideration in view of the following remarks. Issues raised by the Examiner will be addressed below in the order they appear in the prior Office Action. Applicants note for the record that this reply accompanies a Request for Continued Examination (RCE). Applicants' amendments and

remarks are believed to address all of the Examiner's previous concerns, and Applicants contend that this reply places the application in condition for allowance.

1. Applicants note that the amendments filed 24 March 2003 and 26 June 2003 have been entered in full.

2. Applicants note with appreciation that the objection of claims 30, 31, and 39 has been withdrawn.

Applicants note with appreciation that the rejection of claims 13, 15-23, 28-33, 39, 45, 46, 50, 53, 54, 57-60, 76-78, 85, and 87-91 under 35 U.S.C. 112, first paragraph, has been withdrawn in part.

Applicants note with appreciation that the rejection of claims 13, 15-23, 28-33, 39, 45, 46, 50, 51, 53, 54, 57-60, 63, 76-78, and 85 under 35 U.S.C. 112, second paragraph, has been withdrawn.

3. The specification is objected to for an alleged ambiguity with respect to the identity of PYY. Applicants traverse this rejection, and provide the following clarification for any misunderstanding concerning the identity of PYY.

Applicants contend that one of skill in the art would readily appreciate the identity of the PYY polypeptides recited in the application. A functional PYY polypeptide is a 36 amino acid polypeptide (represented in SEQ ID NO: 3). However, this 36 amino acid functional polypeptide is endogenously derived by cleavage of a longer polypeptide represented in SEQ ID NO: 2. This biochemistry of PYY was known at the time of filing of the application, and thus one of skill in the art would readily appreciate that SEQ ID NO: 3 corresponds to PYY, the bioactive, 36 amino acid polypeptide that is generated by cleavage of the precursor polypeptide represented in SEQ ID NO: 2. Applicants have amended the specification to correct any ambiguity. Accordingly, reconsideration and withdrawal of this objection are respectfully requested.

4. Claims 13, 15-23, 28-33, 39, 45, 46, 50, 53, 54, 57-60, 76-78, 85, and 87-95 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for allegedly failing to particularly point out and distinctly claim the subject matter that Applicants regard as the invention.

Applicants traverse this rejection and contend that the rejection is moot in light of the amended claims.

(a) The term "encodable" is rejected as allegedly indefinite. Applicants have amended the claims as suggested by the Examiner. Applicants' amendments merely clarify the scope of the claims and do not narrow their scope. Reconsideration and withdrawal of this rejection are respectfully requested.

(b) Recitation of "altering the glucose-responsiveness" is rejected as allegedly indefinite. Applicants contend that the term is readily appreciated in light of the specification as a whole. Nevertheless, to expedite prosecution of claims directed to commercially relevant subject matter, Applicants have amended the claims to more particularly point out certain embodiments of the invention. Applicants' amendments are not in acquiescence to the rejection, and Applicants reserve the right to prosecute claims of similar or differing scope. Reconsideration and withdrawal of this rejection are respectfully requested.

(c) Recitation of "maintaining or restoring a function of pancreatic β cells or islets" is rejected as allegedly indefinite. Applicants contend that the term is readily appreciated in light of the specification as a whole. Nevertheless, to expedite prosecution, Applicants have amended the claims to more particularly point out certain embodiments of the invention. Applicants' amendments are not in acquiescence to the rejection, and Applicants reserve the right to prosecute claims of similar or differing scope. Reconsideration and withdrawal of this rejection are respectfully requested.

(d) Claims 87 and 92, 88 and 93, 89 and 94, and 91 and 95 are indefinite for reciting the same method. Applicants apologize for this inadvertent error, and have amended the claims to avoid duplication of subject matter among the pending claims. Applicants' amendments are made solely to correct this clerical error, and should not be construed as limiting the scope of the claims so amended. Reconsideration and withdrawal of this rejection are respectfully requested.

5. Claims 13, 15-20, 21-23, 28-33, 39, 45, 46, 50, 53, 54, 57-60, 76-78, and 85 are rejected under 35 U.S.C. 112, first paragraph, for allegedly failing to enable one of skill in the art to

practice the claimed invention. Applicants traverse this rejection and contend that the rejection is moot in light of the amended claims.

The Examiner previously cited Wells to support the notion that because changes in primary sequence can affect the function of a protein or peptide, Applicants are not entitled to claims directed to the use of a broader range of PYY agonists. In response to this argument, Applicants raise the following two points. First, even if the claims were to encompass certain inoperative embodiments, it does not undermine the enablement of the operative subject matter. In accordance with MPEP 2164.08(b), "[t]he presence of inoperative embodiments within the scope of a claim does not necessarily render a claim nonenabled. The standard is whether a skilled person could determine which embodiments that were conceived, but not yet made, would be inoperative or operative with expenditure of no more effort than is normally required in the art." This standard has been upheld in the courts, and permits a claim to encompass a finite number of inoperable embodiments so long as inoperable embodiments can be determined using methodology specified in the application without undue experimentation. See, for instance, *In re Angstadt*, 190 U.S.P.Q. 214 (CCPA 1976). Applicants additionally note however, that given the functional limitations expressly recited in the claims, PYY variant peptides that do not retain the recited function do not fall within the scope of the claims.

Secondly, Applicants point out that the Wells reference relied upon by the Examiner was published in 1990. Since that time, there has been a veritable explosion in the art of combinatorial chemistry which can allow the making and testing of polypeptide variants without undue experimentation. Thus, even if one agrees that small differences in polypeptide sequence can affect the function of a protein or peptide, this point is immaterial in assessing the enablement of the claimed methods. Rather, the important consideration in determining whether Applicants have enabled the use of PYY variants in the subject methods is whether one of skill in the art could readily make and test polypeptide variants using the teachings of the specification and the state of the art, without undue experimentation, in order to select PYY variants for use in the subject methods. Applicants contend that this burden has been met.

The specification provides a detailed description of methods of making and testing variants using combinatorial mutagenesis (page 22, line 24-page 23, line 17). Furthermore, as

noted above, the specification provides mouse models in which PYY variants can be tested for efficacy in the subject methods. Given the extensive guidance provided in the specification, as well as the high level of skill in the art, Applicants contend that one of skill in the art can readily make and test PYY variants to identify variants which meet the structural and functional limitations recited in the claims without undue experimentation.

Furthermore, Applicants have amended the claims to provide additional functional limitations to describe the claimed subject matter. The extensive structural and functional description of the claimed subject matter readily permits one of skill in the art to envision the claimed subject matter, and furthermore to make and use the claimed subject matter. In light of the extensive guidance provided by the specification, the high level of skill in the art **at the time** of filing, and the structural and functional guidance provided by the specification, Applicants contend that the claims are enabled throughout their scope.

Additionally however, Applicants do not merely rely upon the ability of one of skill in the art to make and test peptide variants in order to select variants for use in the methods of the present invention. Applicants reiterate the arguments of record, and remind the Examiner that several PYY variants have been identified and the ability of these variants to mimic one or more functions of PYY has been demonstrated. Accordingly, these examples demonstrate that not only **could** one of skill in the art make and test variants to identify those variants with particular functional attributes, but one of skill in the art **did** make and test variants to identify variants with particular attributes.

Furthermore, evidence gathered since the filing of the present application additionally indicates that one of skill in the art can make and test PYY fragments and variants that retain the functional properties of PYY, as taught by the instant claims. Applicants direct the Examiner's attention to the teachings of Balasubramaniam et al., Liu et al., and Challis et al. (Balasubramaniam et al., 2000, Liu et al. 2001, and Challis et al., 2003; enclosed herewith as Exhibits 1-3).

Balasubramaniam et al. show that several PYY fragments, including a fragment containing only residues 22-36 of the PYY 36 amino acid active polypeptide (a fragment of only approx 1/3 of the total polypeptide), mimic the effects of PYY in vivo in rat intestine. (Exhibit

1). Liu et al. show that several PYY analogs of the 36 amino acid residue polypeptide including PYY (9-36), PYY(14-36), and PYY(22-36) retain the ability to bind to pancreatic cancer cells. (Exhibit 2). We note that the peptide analogs were additionally modified with biotin. This further demonstrates the high level of skill in the art of making and testing polypeptide variants and analogs, and that the making and testing of variants that retain the functional properties of PYY are enabled by the presently claimed invention. Challis et al. present in vivo results demonstrating that the PYY(3-36) variant mimics the effects of PYY(1-36) on food uptake and hypothalamic expression when administered intraperitoneally to mice. (Exhibit 3).

Applicants contend that in light of the teachings of the specification, the structural and functional language recited in the claims, and the high level of skill in the art, Applicants' claims are enabled throughout their scope. Reconsideration and withdrawal of this rejection is respectfully requested.

## CONCLUSION

In view of the foregoing amendments and remarks, Applicants submit that the pending claims are in condition for allowance. Early and favorable reconsideration is respectfully solicited. The Examiner may address any questions raised by this submission to the undersigned at 617-951-7000. Should an extension of time be required, Applicants hereby petition for same and request that the extension fee and any other fee required for timely consideration of this submission be charged to **Deposit Account No. 18-1945.**

Respectfully Submitted,

Date: March 22, 2004

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# Structure−Activity Studies Including a Ψ($CH_2$-NH) Scan of Peptide YY (PYY) Active Site, PYY(22−36), for Interaction with Rat Intestinal PYY Receptors: Development of Analogues with Potent in Vivo Activity in the Intestine#

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Peptide YY (PYY) is a gut hormone that inhibits secretion and promotes absorption and growth in the intestinal epithelium. We have performed structure−activity studies with the active site, N-α-Ac-PYY(22−36)-$NH_2$, for interaction with intestinal PYY receptors. Investigation of aromatic substitutions at position 27 resulted in analogues that exhibited potent in vitro antisecretory potencies with N-α-Ac-[$Trp^{27}$]PYY(22−36)-$NH_2$ exhibiting even greater potency than intact PYY. In vivo studies in dogs revealed that this analogue also promoted intestinal absorption of water and electrolytes during continuous intravenous and intraluminal infusion. Investigations carried out to identify features that would enhance stability revealed that incorporation of $Trp^{30}$ increased affinity for PYY receptors. A "$CH_2$-NH" scan revealed that incorporation of reduced bonds at position 28−29 or 35−36 imparted greater receptor affinity. In general, disubstituted analogues designed based on the results of single substitutions exhibited good receptor affinity with N-α-Ac-[$Trp^{27}$,$CH_2$-$NH^{35-36}$]PYY(22−36)-$NH_2$ having the greatest affinity ($IC_{50}$ = 0.28 nM). Conservative multiple substitutions with Nle→Leu and Nva→Val also imparted good affinity. An analogue designed to encompass most of the favored substitutions, N-α-Ac-[$Nle^{24,28}$,$Trp^{30}$,$Nva^{31}$,$CH_2$-$NH^{35-36}$]PYY(22−36)-$NH_2$, exhibited a proabsorptive effect in dogs comparable to, but longer lasting than, that of intact hormone. Selected analogues also exhibited good antisecretory potencies in rats with N-α-Ac-[$Trp^{30}$]PYY(22−36)-$NH_2$ being even more potent than PYY. However, the potencies did not correlate well with the PYY receptor affinity or the proabsorptive potencies in dogs. These differences could be due to species effects and/or the involvement of multiple receptors and neuronal elements in controlling the in vivo activity of PYY compounds. PYY(22−36) analogues exhibited good affinity for neuronal Y2 receptors but poor affinity for Y1 receptors. Also, crucial analogues in this series hardly bound to Y4 and Y5 receptors. In summary, we have developed PYY(22−36) analogues which, via interacting with intestinal PYY receptors, promoted potent and long-lasting proabsorptive and antisecretory effects in in vivo models. These compounds or analogues based on them may have useful clinical application in treating malabsorptive disorders observed under a variety of conditions.

## Introduction

Peptide YY (PYY) is a 36-residue peptide amide first isolated from porcine intestine.[1] PYY is essentially a gut hormone mainly localized in the endocrine cells of colon, rectum, and ileum.[2,3] Nevertheless, small quantities of PYY have also been found in the enteric nerves surrounding the stomach and in rat brain.[4] PYY, which is released into circulation in response to feeding,[5,6] exhibits a variety of effects on the mammalian gastrointestinal tract. These effects include inhibition of gastric acid and exocrine pancreatic secretions,[1] delaying of gastric emptying,[7] slowing of intestinal transit,[8−9] enhancement of basal and postprandial absorption,[10−13] and inhibition of basal and secretagogue-induced intestinal secretions.[14−19] The PYY homologue, neuropeptide Y (NPY), mimics these activities in the intestine but is 5−10-fold less potent than PYY.[14,15,20] In addition, PYY, but not NPY, promotes intestinal growth in normal rats[21] as well as those maintained on parenteral nutrition.[22] Moreover, plasma PYY levels and its mucosal mRNA levels are elevated in certain malabsorptive disorders.[23,24] These findings suggest that PYY may play a significant role in the physiological regulation of the mammalian gastrointestinal tract.

A PYY receptor system coupled negatively to adenylyl

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Exhibit 1

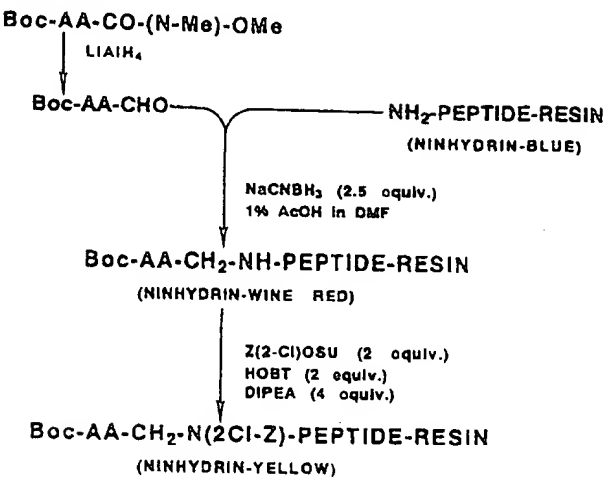
cyclase has been characterized in rat intestinal crypt epithelial cells.[20,25,26] It appears that this receptor may mediate the effects of PYY on intestinal absorption, secretion, and growth.[20] PYY receptors exhibit high affinity for PYY and NPY but bind poorly to pancreatic polypeptide (PP). These same receptors also exhibit high affinity to the C-terminal fragments of NPY and PYY. The latter finding suggests that PYY receptors have characteristics of classical neuronal Y2 receptors of the NPY−PYY−PP family, which also bind well to NPY, PYY, and their C-terminal fragments. On the other hand, typical Y1 receptors bind well only to intact PYY and NPY (see ref 27 for a review). However, elegant investigations by Cox and co-workers have shown that several potent Y2 agonists, including cyclic truncated analogues of NPY[28] and PYY[29] as well as several Ala-substituted NPY analogues,[30] exhibit only weak to moderate antisecretory activity in rat intestinal preparations. Thus, it appears that intestinal PYY receptors, which remain to be cloned, may represent a novel subclass of Y2 receptors.

Malabsorption, which often results following a loss of a critical mass of gut mucosa, may produce chronic diarrhea, malnourishment, and dehydration. Such conditions affect millions of people worldwide per year. Moreover, no effective therapy exists at present for treating this condition. These observations, and the findings that PYY inhibits intestinal secretion and promotes absorption and growth through specific receptors, suggest that it is possible to dissociate various effects of PYY and develop PYY receptor-selective compounds for treating malabsorptive disorders. Our previous efforts toward this goal resulted in the identification of PYY(22−36), A S L R H Y L N L V T R Q R Y, as the active site for interaction with intestinal PYY receptors[26,31] and demonstrated that N-α-Ac-PYY-(22−36)-$NH_2$ analogues could mimic the antisecretory effects of PYY in rat jejunal membrane preparations.[32] In continuation of these investigations, we have now performed further studies with N-α-Ac-PYY(22−36)-$NH_2$ to improve the affinity, selectivity, and in vivo activity. These investigations that are described in this manuscript have resulted in the development of analogues exhibiting long-lasting and potent in vivo effects on intestinal absorption and secretion.

## Results and Discussion

The PYY(22−36) analogues with standard substitutions were synthesized by t-Boc solid-phase method and purified by reversed-phase chromatography in good yields following our previously published procedures.[33,34] To synthesize PYY(22−36) analogues with a $CH_2$−NH bond, we made use of the elegant methods developed by Fehrentz and Castro[35] and by Saski and Coy[36] to obtain optically pure Boc-AA-CHO and couple it directly to the α-amino group of the peptide-resin, respectively. However, the possibility of branching at the IIry amino group has prevented general applicability of this method, especially for the synthesis of long peptides containing a $CH_2$−NH bond in the C-terminal region as in PYY-(22−36). Moreover, this method is not compatible with solid-phase synthesis involving capping procedures because the unprotected IIry amine of $CH_2$-NH is also prone to acetylation. In fact, during our initial attempts

**Scheme 1**




to synthesize N-α-Ac-[$CH_2$-$NH^{35-36}$]PYY(22−36)-$NH_2$ the major product had a mass corresponding to a compound with two acetyl groups, possibly due to the formation of N-α-Ac-[$CH_2$-N-$Ac^{35-36}$]PYY(22−36)-$NH_2$. To ensure no such problems were encountered, we investigated the possibility of temporarily protecting the IIry amine in $CH_2$-NH with Tos, Z, or (2-Cl)Z which could be simultaneously removed during the final HF cleavage to obtain the free peptide. Initial investigations revealed that the treatment of peptide-resin containing $CH_2$-NH with Tos-Cl or Z-Cl in the presence of DIPEA resulted in the complete capping of the IIry amine within 30 min. The red wine color of ninhydrin with IIry amine turned yellow at the end of the capping reaction. However, the known lability of the Z group during repeated acidolysis to remove N-α-Boc groups, and the apparent stability of the Tos group attached to the IIry amine to HF, led us to choose the (2-Cl)Z group for the temporary protection of $CH_2$-NH. This protection was introduced according to Scheme 1 by reacting the N-α-Boc-AA-$CH_2$-NH-peptide-resin with (2-Cl)Z-OSu in the presence of HOBT and DIPEA and monitoring the reaction with ninhydrin. As judged by analytical HPLC, the crude peptides obtained using this strategy contained 70−90% of the target peptides. This procedure has additional advantages including: (1) it could be adapted for automated procedures and (2) it precluded the formation of deletion peptides as the excess of (2-Cl)Z used will also cap the α-amino group, which has not undergone reductive alkylation. All the peptides used in this study had the expected amino acid composition and mass (see Supporting Information) and were >95% homogeneous by analytical HPLC.

Previous investigations have shown that Phe→Tyr[27] substitution in PYY(22−36) increased intestinal antisecretory potency.[32] Therefore, we investigated the effect of substituting a series of aromatic hydrophobic residues at this position. These substitutions in general imparted good PYY receptor affinity and exhibited the following order of potency: Thi > Phe > Bip > Trp ~ Tic ~ Bth > Nal > Pcp > Dip (Table 1: analogues **5**−**13**). As is evident, there was no straightforward correlation between the affinities and the hydrophobicity of the analogues. Probably, other factors such as steric hindrance and changes in the conformation might have

Table 1. Affinities of PYY(22–36) Analogues for Intestinal PYY (rat jejunum), Y-1 (SK-N-MC), and Y-2 (SK-N-BE2) Receptors

| no. | compound | $IC_{50}$ (nM) | | |
|---|---|---|---|---|
| | | intestine (SCC[a]) | SK-N-BE2 | SK-N-MC |
| 1 | PYY | 0.14 ± 0.04 (1.0) | 0.07 ± 0.006 | 0.28 ± 0.05 |
| 2 | NPY | 2.00 ± 0.70 (9.0) | 0.32 ± 0.11 | 3.07 ± 0.52 |
| 3 | [Leu$^{31}$,Pro$^{34}$]PYY | 54 ± 9 | 261 ± 89 | 2.69 ± 0.60 |
| 4 | N-α-Ac-PYY(22–36)-$NH_2$ | 0.91 ± 0.11 (35.7) | 1.30 ± 0.50 | >1000 |
| 5 | N-α-Ac-[Phe$^{27}$]PYY(22–36)-$NH_2$ | 2.3 ± 0.6 (15.1) | 1.83 ± 0.82 | 764 ± 398 |
| 6 | N-α-Ac-[Pcp$^{27}$]PYY(22–36)-$NH_2$ | 7.49 ± 5.00 | 8.09 ± 3.35 | >10000 |
| 7 | N-α-Ac-[Thi$^{27}$]PYY(22–36)-$NH_2$ | 1.00 ± 0.10 (100) | 2.63 ± 0.83 | 257 ± 8.5 |
| 8 | N-α-Ac-[Tic$^{27}$]PYY(22–36)-$NH_2$ | 3.40 ± 0.50 | 186 | ND |
| 9 | N-α-Ac-[Bip$^{27}$]PYY(22–36)-$NH_2$ | 2.42 ± 0.90 | 14.2 ± 6.2 | 279 ± 9 |
| 10 | N-α-Ac-[Dip$^{27}$]PYY(22–36)-$NH_2$ | 16.5 ± 8.2 | 10.00 ± 4.22 | 447 ± 281 |
| 11 | N-α-Ac-[Nal$^{27}$]PYY(22–36)-$NH_2$ | 6.60 ± 5.30 (0.1) | 7.08 ± 4.65 | 88.3 ± 17 |
| 12 | N-α-Ac-[Bth$^{27}$]PYY(22–36)-$NH_2$ | 3.99 ± 0.53 (1.0) | 5.4 ± 2.53 | 105 ± 57 |
| 13 | N-α-Ac-[Trp$^{27}$]PYY(22–36)-$NH_2$ | 3.20 ± 1.23 (0.005) | 2.09 ± 1.1 | 76.95 ± 17 |
| 14 | N-α-Ac-[Trp$^{27}$]PYY(22–36)NH-$CH_3$ | >100 | >100 | >1000 |
| 15 | N-α-Ac-[Trp$^{24}$]PYY(22–36)-$NH_2$ | 2.10 ± 0.10 | ND | ND |
| 16 | N-α-Ac-[Trp$^{28}$]PYY(22–36)-$NH_2$ | 0.60 ± 0.30 (5.00) | 2.08 ± 0.79 | 398 ± 116 |
| 17 | N-α-Ac-[Trp$^{29}$]PYY(22–36)-$NH_2$ | 100 | 158 ± 83 | 762 ± 537 |
| 18 | N-α-Ac-[Trp$^{30}$]PYY(22–36)-$NH_2$ | 0.60 ± 0.30 (2.00) | 0.98 ± 0.26 | 250 ± 207 |
| 19 | N-α-Ac-[D-Trp$^{30}$]PYY(22–36)-$NH_2$ | >100 | 56.5 ± 9.8 | 47.9 ± 10.619. |
| 20 | N-α-Ac-[Nal$^{30}$]PYY(22–36)-$NH_2$ | 3.54 ± 0.72 | 1.09 ± 0.17 | 159 ± 56 |
| 21 | N-α-Ac-[Bth$^{30}$]PYY(22–36)-$NH_2$ | 1.12 ± 0.24 | 3.3 ± 1.79 | 103 ± 31 |
| 22 | N-α-Ac-[Dip$^{36}$]PYY(22–36)-$NH_2$ | >100 | 1672 ± 430 | >10000 |
| 23 | N-α-Ac-[$CH_2$-NH$^{35-36}$]PYY(22–36)-$NH_2$ | 0.50 ± 0.08 | 1.32 ± 0.80 | >10000 |
| 24 | N-α-Ac-[$CH_2$-NH$^{33-34}$]PYY(22–36)-$NH_2$ | >100 | >10000 | 1248 ± 495 |
| 25 | N-α-Ac-[$CH_2$-NH$^{32-33}$]PYY(22–36)-$NH_2$ | 5.03 ± 1.18 | 52 ± 11 | 1091 ± 987 |
| 26 | N-α-Ac-[$CH_2$-NH$^{31-32}$]PYY(22–36)-$NH_2$ | 11.89 ± 3.28 | 105 ± 20 | 1597 ± 906 |
| 27 | N-α-Ac-[$CH_2$-NH$^{30-31}$]PYY(22–36)-$NH_2$ | >100 | >3000 | 769 ± 125 |
| 28 | N-α-Ac-[$CH_2$-NH$^{28-29}$]PYY(22–36)-$NH_2$ | 0.36 ± 0.10 | 55 ± 14 | 1390 |
| 29 | N-α-Ac-[$CH_2$-NH$^{27-28}$]PYY(22–36)-$NH_2$ | >100 | 864 | 1270 |
| 30 | N-α-Ac-[$CH_2$-NH$^{25-26}$]PYY(22–36)-$NH_2$ | 4.22 ± 1.23 | 41 ± 14 | >1000 |
| 31 | N-α-Ac-[$CH_2$-NH$^{24-25}$]PYY(22–36)-$NH_2$ | 2.76 ± 0.98 | 59 ± 19 | 735 ± 147 |
| 32 | N-α-Ac-[Phe$^{27}$,Thi$^{36}$]PYY(22–36)-$NH_2$ | 4.50 ± 1.50 | ND | ND |
| 33 | N-α-Ac-[Phe$^{27,36}$]PYY(22–36)-$NH_2$ | 14.10 ± 5.30 | 1.16 ± 0.51 | >10000 |
| 34 | N-α-Ac-[Phe$^{22,27}$]PYY(22–36)-$NH_2$ | 11.20 ± 3.18 | 5.61 ± 3.02 | 275 ± 76 |
| 35 | N-α-Ac-[Tyr$^{22}$,Phe$^{27}$]PYY(22–36)-$NH_2$ | 3.40 ± 2.6 | 2.68 ± 1.38 | 48.9 ± 7.2 |
| 36 | N-α-Ac-[Ala$^{26}$,Phe$^{27}$]PYY(22–36)-$NH_2$ | 5.30 ± 2.7 | 3.84 ± 2.04 | 674 ± 616 |
| 37 | N-α-Ac-[Trp$^{28,30}$]PYY(22–36)-$NH_2$ | 1.07 ± 0.13 | 3.17 ± 0.51 | 233 ± 68 |
| 38 | N-α-Ac-[Trp$^{30}$,$CH_2$-NH$^{35-36}$]PYY(22–36)-$NH_2$ | 0.28 ± 0.07 | 1.39 ± 0.53 | 1514 ± 514 |
| 39 | N-α-Ac-[Trp$^{27}$,$CH_2$-NH$^{35-36}$]PYY(22–36)-$NH_2$ | 1.12 ± 0.34 | 1.05 ± 0.26 | >1000 |
| 40 | N-α-Ac-[Nle$^{24,28,30}$,Nva$^{31}$]PYY(22–36)-$NH_2$ | 0.59 ± 0.02 | 57.0 ± 10.1 | 1586 ± 13 |
| 41 | N-α-Ac-[Nle$^{24,28}$,Trp$^{30}$,Nva$^{31}$]PYY(22–36)-$NH_2$ | 0.56 ± 0.20 | 3.73 ± 0.89 | 183 ± 25 |
| 42 | N-α-Ac-[Nle$^{24,28}$,Trp$^{30}$,Nva$^{31}$,$CH_2$-NH$^{35-36}$]PYY(22–36)-$NH_2$ | 0.31 ± 0.11 | 1.75 ± 0.44 | 1567 ± 304 |
| 43 | bis(24/24′){N-α-Ac-[Cys $^{24}$]PYY(22–36)-$NH_2$} | 0.63 ± 0.27 | 0.39 ± 0.17 | 110 ± 22 |

[a] SCC, short circuit current; antisecretory potency of peptides was determined by measuring short circuit current in rat jejunal membrane preparation clamped in an Ussing chamber (from refs 17 and 32). ND, not determined.

contributed to the net receptor affinity of the analogues. Alkylation of the C-terminal amide group is known to prolong the half-life of peptide hormones.[37] However, in this instance, methylation, as in **14**, substantially decreased the affinity possibly due to steric hindrance at the binding site.

Further SAR studies revealed that substitution of Leu residues in N-α-Ac-PYY(22–36)-$NH_2$ with Trp at 28 or 30 increased the affinity, while substitution at 29 decreased it (analogues **15**–**18**). The increase in affinity with Trp$^{28}$ or Trp$^{30}$ substitutions may be due to the fact that hydrophobic residues are favored at this position, they increase proteolytic stability, or both. In fact, it has been reported that the Asn$^{29}$–Leu$^{30}$ bond is susceptible to cleavage by endopeptidase-24.11.[38] This observation is also in agreement with that of Fauchere and Thurieau,[39] who have suggested that the location of modifications used to stabilize the active sites of a number of polypeptide hormones have been or could have been guided by prior identification of the cleavage sites. Although Trp-like residues, Bth and Nal, were also tolerated at position 30, D-Trp$^{30}$ substitution substantially decreased affinity (analogues **19**–**21**).

The ability of these analogues to inhibit intestinal secretion was then compared by measuring the short circuit current (SCC) in rat jejunal mucosa according to our previously published procedures.[17] Most of these analogues modified at position 27 exhibited greater antisecretory potency than even intact PYY, with N-α-Ac-[Trp$^{27}$]PYY(22–36)-$NH_2$ (**13**) exhibiting the most potent effect (Table 1). However, the antisecretory potencies did not correlate well with the PYY receptor affinities of the analogues. The reasons for the discrepancy remain unclear at present.

Previous studies have shown that repeated treatment of intestinal mucosa with PYY causes desensitization of the receptor system in this tissue as evidenced by reduced SCC response.[17,18] Therefore, we investigated this phenomenon with N-α-Ac-[Trp$^{27}$]PYY(22–36)-$NH_2$ (**13**). In contrast to PYY,[17] this analogue was more effective in inhibiting the SCC response in a cumulative dose–response study than in experiments where each dose was tested in a fresh tissue (Figure 1). Moreover,

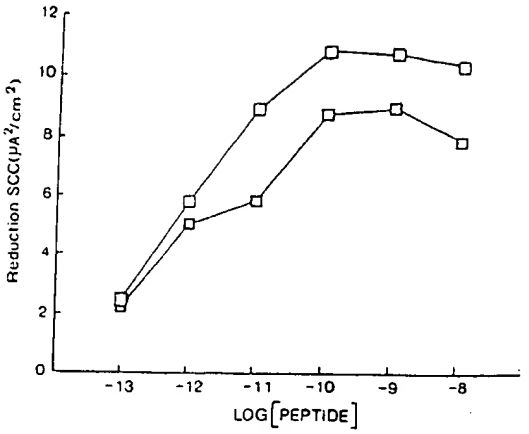



**Figure 1.** Effects of increasing concentrations of N-α-Ac-[Trp$^{27}$]PYY(22–36)-$NH_2$ (**13**) on short circuit current (SCC) in rat jejunal mucosal membranes mounted on Ussing chamber. Dose–response curve obtained using a single tissue which received all the doses cumulatively (□) or a fresh tissue for each dose (□). Each point represents the mean of 8–10 tissues. SE was less than 10% and is not included in the figure for clarity. This is in contrast to PYY where the SCC dose–response curve obtained in cumulative experiment was shifted to the right relative to that obtained with fresh tissue for each dose.[17]

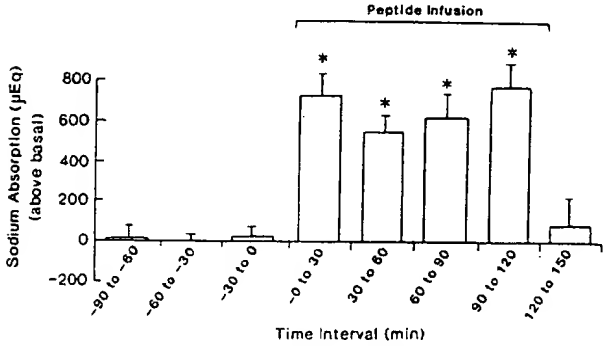



**Figure 2.** Ileal sodium fluxes above basal in six awake dogs with exteriorized jejunal loops infused (iv) with N-α-Ac-[Trp$^{27}$]-PYY(22–36)-$NH_2$ (**13**) (200 pmol/kg/h) for 2 h starting at time zero. Water and chloride absorption paralleled that of sodium; $p < 0.05$ vs basal by ANOVA. Peak effect of **13** is comparable to that of PYY (see Figure 3).

it was determined that the pretreatment of the mucosal preparations with N-α-Ac-[Trp$^{27}$]PYY(22–36)-$NH_2$ (**13**) did not significantly attenuate the SCC response of PYY (100 nM) (not shown). These observations suggest that PYY(22–36) analogues may not cause desensitization observed with the intact hormone. It appears therefore that the N-terminal segment of PYY may also be required to trigger desensitization of PYY receptors.

N-α-Ac-[Trp$^{27}$]PYY(22–36)-$NH_2$ (**13**), being the most potent analogue in this series, was also selected to investigate whether PYY(22–36) analogues could promote intestinal absorption similar to intact PYY during intravenous or intraluminal administration in dogs.[10–12] These experiments were performed in awake dogs with isolated neurovascularly intact ileal loops (Thiry-Villa Fistulas). Intravenous infusion of 200 pmol/kg/h for 2 h significantly increased the ileal absorption of water and electrolytes (e.g., Figure 2). The proabsorptive effects of intraluminal administration of N-α-Ac-[Trp$^{27}$]-PYY(22–36)-$NH_2$ (**13**) in the ileum (200 pmol/kg/h) or colon (300 pmol/kg/h) were also investigated using the same dog model (Table 2). Ileal administration of the analogue significantly enhanced the absorption of water and electrolytes in the ileum and had no effects in colon. Similarly colonic administration only enhanced the absorption in the colon. The confined effects of the analogue within the isolated segment suggest the involvement of PYY receptors on the luminal side. The significance of this observation is that the synthetic analogue could also be adapted for oral delivery using a stable formulation that resists degradation in the gut. However, the proabsorptive effects of both the analogue (Figure 2) and intact PYY$^{10–12}$ reached statistically insignificant levels soon after the iv or intraluminal infusions were stopped. This decrease in effect is not surprising because both PYY and the analogue are known to be susceptible to proteolysis.[38]

**Table 2.** Peak Proabsorptive Effects of Intraluminal Infusion of N-α-Ac-[Trp$^{27}$]PYY(22–36)-$NH_2$ (**13**) in the Ileum and Colon of Awake Dogs Bearing Ileal and/or Colonic Thiry-Vella Fistulas[a]

| | ileum | | colon | |
|---|---|---|---|---|
| | basal | peak[b] | basal | peak[b] |
| $H_2O$ (μL/min) | 437 ± 92 | 725 ± 98 | 204 ± 25 | 503 ± 49 |
| $Na^+$ (μequiv/min) | 68 ± 13 | 120 ± 14 | 41 ± 5 | 78 ± 9 |
| $Cl^-$ (μequiv/min) | 63 ± 9 | 101 ± 13 | 45 ± 5 | 77 ± 8 |

[a] Peptides were infused for 1 h starting at time zero: in ileum at 200 pmol/kg/h and in colon at 300 pmol/kg/h. [b] $p < 0.05$ vs basal by ANOVA. Peak absorptions were observed 30 and 90 min after starting intraluminal infusion in the ileum and colon, respectively.

Further SAR studies were therefore performed to identify features which would enhance the half-life of PYY(22–36) analogues without jeopardizing PYY receptor affinity. Incorporation of a $CH_2$–NH bond has been reported to increase the stability and impart dramatic changes in the property and/or potency of the active sites of a number of polypeptide hormones.[39,40] Therefore, we investigated the effects of incorporating $CH_2$–NH bonds in PYY(22–36) except those involving His, Asn, and Gln (analogues **23–31**).While Asn and Gln are not compatible with this procedure, we had difficulty in preparing Boc-His(Bom)-CHO in good yields. The $CH_2$–NH bonds at 27–28 (**29**) and 33–34 (**24**) substantially reduced the receptor affinity, while those analogues with reduced bonds at 35–36 (**23**) and 28–29 (**28**) exhibited the highest affinity in this series (Table 1). This increase in affinity may be due to increased resistance to proteolysis, easy adaptation of the bioactive conformation due to increased flexibility of the bond, or both. The $CH_2$–NH bond in **28** may also increase the stability of its penultimate bond at 29–30, which is prone to endopeptidase-24.11.[37,39] Analogues with $CH_2$–NH bonds at either 31–32 (**26**) or 32–33 (**25**) also exhibited 10 times greater selectivity for PYY receptors relative to Y2 receptors. Incorporation of $CH_2$–NH bonds at other positions resulted in analogues with moderate affinity (Table 1).

On the basis of the information gathered from analogues **4–31**, we then synthesized analogues incorporating modifications at two sites. Most of these modifications, analogues **32–39**, were tolerated and also resulted in the development of one of the most potent analogues: N-α-Ac-[Trp$^{30}$,$CH_2$-NH$^{35–36}$]PYY(22–36)-$NH_2$

**Table 3.** Potencies of PYY(22–36) Analogues for Inhibiting VIP-Induced Secretion in Isolated Jejunum in Anesthetized Rats[a]

| no. | compound | $ID_{50}$ (pmol/kg) | 95% conf limit |
|---|---|---|---|
| 1 | PYY | 6.0 | 3.9–9.3 |
| 13 | N-α-Ac-[$Trp^{27}$]PYY(22–36)-$NH_2$ | 33.3 | 17.1–64.8 |
| 18 | N-α-Ac-[$Trp^{30}$]PYY(22–36)-$NH_2$ | 2.1 | 0.47–9.44 |
| 38 | N-α-Ac-[$Trp^{30}$,$CH_2$-$NH^{35-36}$]PYY(22–36)-$NH_2$ | 190 | 95–370 |
| 40 | N-α-Ac-[$Nle^{24,28,30}$,$Nva^{31}$]PYY(22–36)-$NH_2$ | 29.5 | 16.6–52.3 |
| 41 | N-α-Ac-[$Nle^{24,28}$,$Trp^{30}$,$Nva^{31}$]PYY(22–36)-$NH_2$ | 13 | 8.7–19.4 |
| 42 | N-α-Ac-[$Nle^{24,28}$,$Trp^{30}$,$Nva^{31}$,$CH_2$-$NH^{35-36}$]-PYY(22–36)-$NH_2$ | 59 | 31–116 |
| 43 | bis(24/24′){N-α-Ac-[$Cys^{24}$]PYY(22–36)-$NH_2$} | 365 | 95–1400 |

[a] VIP was administered at 30 μg/kg/h. Each point was investigated in 6–8 animals.

(38). We also investigated the effects of conservative multiple substitutions with Nle→Leu and Nva→Val because these modifications, if tolerated, would further enhance the proteolytic stability. This analogue, N-α-Ac-[$Nle^{24,28,30}$,$Nva^{31}$]PYY(22–36)-$NH_2$ (40), exhibited high affinity to PYY receptors, but $Trp^{30}$ substitution as in 41 was also essential for potent in vivo activity (Table 3). We then synthesized 42, N-α-Ac-[$Nle^{24,28}$,$Trp^{30}$,-$Nva^{31}$,$CH_2$-$NH^{35-36}$]PYY(22–36)-$NH_2$ encompassing all the modifications in 38, 40, and 41. This analogue exhibited an affinity comparable to the most potent analogue, #38, and in preliminary studies exhibited greater proteolytic stability than 38, 40, and 41. Therefore, we tested the in vivo proabsorptive effects of 42 in dogs in collaboration with Townsend and co-workers.[41] During iv infusions, this analogue promoted intestinal absorption to a level comparable to that of PYY, and as expected its peak effects were longer lasting than those of PYY (Figure 3).

Since dimerization of the active sites has been reported to increase potency and/or stability,[42] we synthesized the PYY(22–36) dimer via $Cys^{24}$ (43). This dimerization did not improve the receptor affinity very much, compared to its monomer (Table 1). It is possible that this may not be the optimal dimerization site. Further investigations are therefore necessary to determine whether this approach will yield potent PYY-(22–36) analogues.

PYY has been shown to inhibit basal and secretagogue-induced intestinal secretion in a number of animal models including humans.[14–19] Therefore, we selected those analogues exhibiting high affinity to PYY receptors and compared their ability to inhibit intestinal secretion with that of PYY in anesthetized rats, according to our previously published procedures.[15] In this study, 30-min inhibitory effects of bolus doses (iv) of PYY(22–36) analogues on VIP (30 μg/kg/h)-induced intestinal secretion were investigated. All these analogues dose-dependently inhibited the intestinal secretion with varying degree of potencies (Table 3). N-α-Ac-[$Trp^{30}$] PYY(22–36)-$NH_2$ (18), which is stable to endopeptidase-24.11, exhibited potent antisecretory activity, greater than even that of intact PYY. On the other hand, although both N-α-Ac-[$Nle^{24,28}$,$Trp^{30}$,$Nva^{31}$]PYY(22–36)-$NH_2$ (41) and N-α-Ac-[$Nle^{24,28,30}$,$Nva^{31}$]PYY(22–36)-$NH_2$ (40) are stable to endopeptidase-24.11 and exhibit comparable affinity to PYY receptors, 41 exhibited greater antisecretory activity. This observation suggests that $Trp^{30}$ plays a greater role than just imparting proteolytic stability. N-α-Ac-[$Trp^{27}$]PYY(22–36)-$NH_2$

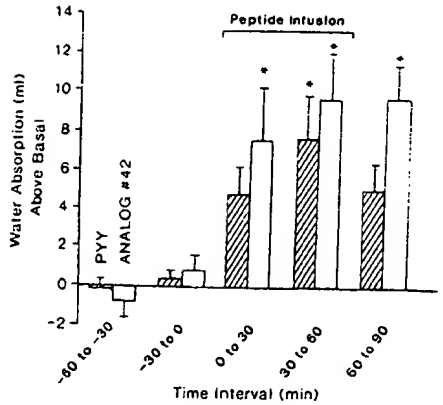



**Figure 3.** Comparison of the ileal proabsorptive effects of N-α-Ac-[$Nle^{24,28}$,$Trp^{30}$,$Nva^{31}$,$CH_2$-$NH^{35-36}$]PYY(22–36)-$NH_2$ (42) with that of PYY in six awake dogs with exteriorized jejunal loops. Each compound was infused (iv) at the rate of 200 pmol/kg/h for 60 min starting at time zero; $p < 0.05$ vs basal by ANOVA. Peak effect of 42 was sustained for a longer period than that of PYY.

(13) and N-α-Ac-[$Nle^{24,28}$,$Trp^{30}$,$Nva^{31}$,$CH_2$-$NH^{35-36}$]PYY-(22–36)-$NH_2$ (42), which exhibited potent and/or long-lasting proabsorptive effects in dogs, were only moderately potent as antisecretory agents. Moreover, N-α-Ac-[$Trp^{30}$,$CH_2$-$NH^{35-36}$]PYY(22–36)-$NH_2$ (38), which exhibited the greatest PYY receptor affinity, was one of the weakest in this series. So was the dimer, 43. As is always the case with in vivo experiments, the antisecretory data cannot be interpreted based solely on PYY receptor affinity. This disparity could be due to many factors including differences in in vivo stability, accessibility to receptors, species differences, and involvement of multiple receptors. Moreover, one cannot exclude the involvement of neuronal factors, at least in part, in controlling PYY effects on intestinal secretion.[16] These results, together with future time course experiments, may prove useful in identifying potent and long-acting antisecretory analogues.

While our studies were in progress, cloning of additional receptor subtypes, Y4 and Y5, of the NPY–PYY–PP family of hormones was reported (see ref 27 for a review). Moreover, one of us (M.L.) has recently determined that Y2, Y4, and Y5 receptors are also present in colonic or intestinal mucosa.[43] Although these receptors may also be involved in mediating the effects of PYY peptides in the intestine, our recent investigation with crucial analogues in this series, N-α-Ac-[$Trp^{27}$]-PYY(22–36)-$NH_2$ (13), N-α-Ac-[$Trp^{30}$]PYY(22–36)-$NH_2$ (18), and N-α-Ac-[$Nle^{24,28}$,$Trp^{30}$,$Nva^{31}$,$CH_2$-$NH^{35-36}$]PYY-(22–36)-$NH_2$ (42), revealed that they exhibit lower affinity ($IC_{50}$ > 1000 nM) to Y4 and Y5 receptors (not shown). Moreover, these analogues bound poorly to Y1 receptors (Table 1). These observations are consistent with our previous results that PYY(22–36) analogues do not cause hypertension,[31] an effect predominantly mediated by Y1 receptors. On the other hand, PYY(22–36) analogues, with few exceptions, also bound well to Y2 receptors (Table 1). It appears therefore that Y2 receptors, in addition to PYY receptors, may also be involved in the in vivo effects of PYY(22–36) analogues. Alternatively, since PYY receptors have not been cloned yet, we cannot strictly rule out the possibility that they represent a peripheral form of Y2 receptors in intestine.

Although one could not rule out these possibilities, the following observations suggest otherwise: (1) PYY exhibited a more pronounced effect than NPY on actin reorganization and induction of CD63 and clustering transcripts in an intestinal epithelial cell line;[44] (2) PYY, and not NPY, enhanced intestinal growth;[21] (3) intestinal distributions of Y2 receptor mRNAs are different from those of functional PYY receptors;[43] (4) potent Y2 agonists exhibited poor antisecretory potencies.[28–30] Experiments with receptor knock-out models and receptor-antisense and receptor-selective ligands may shed light on whether different receptors are involved in mediating PYY effects in the intestine.

The observation that some of the potent Y2 agonists elicit weak effects on PYY receptor-mediated antisecretory functions and the finding that analogues **25**, **26**, and **40** were 10–100 times selective to rat intestinal PYY receptors relative to human neuroblastoma Y2 receptors (Table 1) suggest that it is possible to dissociate Y2 activity from PYY(22–36) analogues and develop ligands highly selective for intestinal PYY receptors. However, apparently no adverse effects were observed with the PYY(22–36) analogues in the animal models used in this study. Therefore, one questions whether there is a need to dissociate Y2 activity. Moreover, the peripheral adverse effects, if any, could be avoided via oral delivery, because analogues delivered luminally do not appear to enter into circulation in the active form.

In summary, we have developed analogues that bind to intestinal PYY receptors and promote potent and long-lasting in vivo effects on intestinal absorption and secretion. Although it remains to be proven, these compounds may also promote repletion of intestine similar to intact PYY. Therefore, it appears that these compounds or analogues based on them may prove useful in treating malabsorptive disorders observed under a variety of conditions including chronic diarrhea, short bowel syndrome, and intestinal bowel diseases.

## Experimental Section

**Materials.** N-α-Boc-amino acids with benzyl-based protecting groups (Midwest Biotech, Indianapolis, IN) and peptide synthesis reagents (Applied Biosystem, Inc., Foster City, CA) and solvents (Tedia Inc., Cincinnati, OH) were obtained commercially and used without further purification. All protease inhibitors and buffer reagents were purchased from Sigma Chemical Co., St. Louis, MO, Biowhitaker, Walkersville, MA, and/or Life Technologies, Grand Island, NY. Neuroblastoma cells, SK-N-MC and SK-N-BE2 cells, were gifts from Dr. June Biedler, Sloan–Kettering Memorial Institute, NY. Sources of rats and other reagents have been reported previously.[33,34]

**Peptide Synthesis.** Peptides were synthesized according to our previously published procedures for the synthesis of NPY.[33] Briefly, the protected amino acids were assembled sequentially on *p*-methylbenzhydrylamine resin (0.45 mmol amino group) using an automated Applied Biosystem instrument employing a program supplied by the manufacturers for single coupling procedures. All amino acids were coupled using 4.4 equiv of pre-formed 1-hydroxybenzotriazole esters.

**Synthesis of Peptides Containing $CH_2$–NH Bonds.** Coupling of t-Boc-amino acid aldehydes was performed manually as described by Saski and Coy.[36] Briefly, t-Boc-amino acid aldehyde (4 equiv), obtained by $LiaAlH_4$ reduction of the corresponding *N*-methoxy-*N*-methylamide derivatives,[35,45] was reacted immediately with the free α-amino group resin containing the appropriate peptide sequence with protected side chains and in DMF containing 1.0% HOAC in the presence of an equivalent quantity of $NaBH_3CN$. At the end of the reaction (2–3 h), the formation of the secondary amino group was confirmed with ninhydrin (wine-red color). To prevent acylation of the $CH_2$–NH bond during subsequent coupling or capping steps, the peptide-resin with the secondary amine was reacted with 2 equiv of Z(2-Cl)OSU, 2 equiv of HOBT, and 4 equiv of diisopropylethyamine until the ninhydrin test gave a yellow color. Automated synthesis was then resumed by reintroducing the peptide-resin into the recation vessel of the synthesizer.

**Synthesis of Dimers.** Peptides were dimerized on the resin by coupling 0.5 equiv of Boc-Cys in the presence of equivalent quantities of HBTU, HOBT and DIEA. This coupling which was also performed manually was generally complete within 10–18 h. In some instances, the uncoupled amino group was capped by acetylation. Stepwise synthesis was then continued to obtain the desired sequences. If required, the α-amino group was acetylated at the end of the synthesis.

**HF Cleavage.** At the end of the synthesis, the N-α-Boc group was removed as programmed, and if required the α-amino group was acetylated automatically with $Ac_2O$. $N^{in}$-CHO, if present, was then removed with 20% piperidine–DMF, and the free peptides were obtained by treating the peptide-resins (~0.25 mmol) with HF (~10 mL) containing 5% *p*-cresol for about 1 h at −2 to −4 °C. In the case of peptides with Trp or Cys, HF reaction mixture also contained ~2.5% dimethyl sulfide. The residue after HF cleavage was washed repeatedly with diethyl ether, and then extracted with 30% acetic acid (2 × 15 mL), diluted to 10% and lyophilized.

**Purification.** Peptides were purified according to our previously published procedures for NPY,[33] using a Waters Instrument with model 600 multisolvent delivery system, U6K injector, model 481 spectrphotometer and Baseline 810 data collection. Analytical HPLC was performed on a Vydac C18 column (250 × 4.6 mm, 5-μm particle size, 300-Å pore size) at a flow rate of 1 mL/min. Semipreparative HPLC was carried out on a Vydac column (250 × 10 mm, 10-μm particle size, 300-Å pore size) at a flow rate of 4.7 mL/min. Detection was at 214 nm. A combination of either 0.1% TFA–$H_2O$ (A) and 60% MeCN in A (B) or 0.1% triethylyammonium phosphate buffer, pH 2.25 (C) and 60% MeCN in C (D) were used to purify the peptides. If sovent system C and D were used, peptides were desalted using A and B, before lyophilization. All the purified peptides were characterized by analytical reversed-phase chromatography using an isocratic gradient and amino acid and mass spectral analyses.

**Cell Culture.** The neuroblastoma cells were grown in Eagle's essential medium (EMEM), supplemented with 10% fetal calf serum, penicillin (100 IU/mL) and streptomycin (50 μg/mL) in a 95% air–5% $CO_2$ humidified atmosphere at 37 °C. When the cells became 90% confluent (4–5 days), the growth medium was aspirated, cells washed with Dulbecco's phosphate-buffered saline (DPBS) with $Ca^{2+}$ and $Mg^{2+}$, then dispersed by incubating with 0.1% trypsin in DPBS, pH 7.4, without $Ca^{2+}$ and $Mg^{2+}$ for 5 min at 37 °C.

**Receptor Binding.** The SK-N-MC and SK-N-BE2 cell lines were cultured in EMEM media containing 10% fetal calf serum and 5% chicken embryo extract in a humidified atmosphere (37 °C) of 95% air and 5% $CO_2$. The appropriate cells were harvested, homogenized (Polytron, setting 6, 15 s) in ice-cold 50 mM Tris-HCl (buffer A), and centrifuged twice at 39000*g* (10 min), with an intermediate resuspension in fresh buffer. The final pellets were resuspended in 50 mM Tris-HCl, containing 0.1 mg/mL bacitracin, and 0.1% BSA (buffer B), and held on ice for the receptor binding assay. Aliquots (0.4 mL) were incubated with 0.05 mL of [$^{125}$I]PYY (SK-N-MC cells) or [$^{125}$I]PYY(3–36) (SK-N-BE2) (~2200 Ci/mmol, New England Nuclear) and buffer B, with and without 0.05 mL of unlabeled competing peptides. After a 120-min incubation (25 °C), the bound [$^{125}$I]PYY or [$^{125}$I]PYY(3–36) was separated from the free by rapid filtration through GF/C filters previously soaked in 0.3% poly(ethylenimine). The filters were then washed three times with 5-mL aliquots of ice-cold buffer A. Specific binding was defined as the total [$^{125}$I]PYY bound minus that bound in the presence of intact PYY.

**Binding Studies with Rat Intestinal Crypt Epithelial membranes.** This was performed according to our previously published procedures.[25,26] Jejunal segments were removed and flushed free of content, filled with 0.34 M NaCl. Crypt cells were separated from villus cells by shaking the everted jejunum for successive periods in a dispersing solution containing 2.5 mM EDTA and 0.24 M NaCl, pH 7.5. Villus cells were released first and crypt cells after a prolonged shaking. Crypt cells were then sedimented at 2000*g* for 2 min and washed 4 times with Krebs-Ringer phosphate buffer, pH 7.5. Crude membranes were prepared from isolated crypt cells as described.[22,26] Displacement studies were performed in a total volume of 0.25 mL of 20 mM HEPES assay buffer, pH 7.4, containing 2% bovine serum albumin and 0.1% bacitracin. In a standard assay 200 $\mu$g of membrane protein/tube were incubated for 2 h at 15 °C in a shaking water bath with [$^{125}$I]-PYY (50 pM) and increasing concentrations of peptides. At the end of incubation tubes were vortexed and 150-$\mu$L aliquots transferred into polypropylene tubes containing 250 $\mu$L of ice-cold assay buffer. Unbound [$^{125}$I]PYY were separated by centrifugation at 20000*g* for 10 min followed by aspiration of the supernatant. The tubes containing the pellet were counted for bound radioactivity in a Micromedic gamma counter. Each point in an experiment was carried out in triplicate and the experiment repeated at least three times. Displacement curves were plotted using LIGAND program and the $IC_{50}$ values determined.

**In Vivo Experiments in Rats To Determine the Effects of PYY Analogues on VIP-Induced Jejunal Secretions.** This was carried out according to the procedures developed by Roze and co-workers.[15] Briefly, rats were anesthetized, and a saphenous vein was cannulated to infuse peptides. The abdomen was opened, and a jejunal loop (~20 cm long) was delimited by two ligatures. At time zero, 2 mL of 0.9% saline prewarmed to 37 °C was instilled into the jejunal loop. The loop was then returned into the abdomen and closed. VIP (30 $\mu$g/kg/h) was infused through the saphenous vein at the rate of 2.5 mL/h for 30 min starting at time zero. PYY analogues (3–900 pmol/kg) or saline (controls) was injected as bolus doses 15 min before starting VIP infusions. At the end of the experiment rats were sacrificed, and the jejunal loop excised. The loop was then measured and weighed before and after removing the instilled fluid. The net water flux expressed as $\mu$L/cm/30 min was calculated using the formula $(F - E) - 2000/L$, where $F$ and $E$ are the weights of the loop before and after emptying the remaining fluid, $L$ is the length of the loop in cm, and 2000 is the volume in $\mu$L of the fluid instilled initially. Net absorption is indicated by a negative value and net secretion by a positive value. Each dose was investigated in 6–8 animals.

**In Vivo Experiments in Dogs To Determine the Effects of PYY Analogues on Intestinal Absorption.** In vivo effects of peptides on water and electrolyte absorption by the jejunum, ileum and colon under basal conditions were investigated in awake dogs with jejunal, ileal and/or colonal Thiry-Vella loops according to our previously published procedures.[10–12] Each experiment consisted of a 90-min basal period followed by a 150-min experimental period. The small bowel segment was perfused through the proximal cannula at 2 mL/min using a roller pump. The perfusate, pH 7.4 maintained at 37 °C, contained (in nmol/L) 140 $Na^+$, 5.2 $K^+$, 119.8 $Cl^-$, 25 $HCO_3^-$, 1.2 $Ca^{2+}$, 1.2 $Mg^{2+}$, 2.4 $HPO_2^{4-}$, 0.4 $H_2PO_4^-$, 10 glucose and 10 $\mu$C [$^{14}$C]poly(ethylene glycol) in 5 g/L poly(ethylene glycol). After a 20-min washout period, perfusate was collected every 15 min for determination of $Na^+$, $Cl^-$ and water contents.

**Supporting Information Available:** HPLC, MS, and amino acid analysis data of PYY(22–36) analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACS PUBLICATIONS

# Structure-activity studies including a Psi(CH(2)-NH) scan of peptide YY (PYY) active site, PYY(22-36), for interaction with rat intestinal PYY receptors: development of analogues with potent in vivo activity in the intestine.

**Balasubramaniam A, Tao Z, Zhai W, Stein M, Sheriff S, Chance WT, Fischer JE, Eden PE, Taylor JE, Liu CD, McFadden DW, Voisin T, Roz C, Laburthe M.**

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Peptide YY (PYY) is a gut hormone that inhibits secretion and promotes absorption and growth in the intestinal epithelium. We have performed structure-activity studies with the active site, N-alpha-Ac-PYY(22-36)-NH (2), for interaction with intestinal PYY receptors. Investigation of aromatic substitutions at position 27 resulted in analogues that exhibited potent in vitr antisecretory potencies with N-alpha-Ac-[Trp(27)]PYY(22-36)-NH(2) exhibiting even greater potency than intact PYY. In vivo studies in dogs revealed that this analogue also promoted intestinal absorption of water and electrolytes during continuous intravenous and intraluminal infusion. Investigations carried out to identify features that would enhance stability revealed that incorporation of Trp(30) increased affinity for PYY receptors. A "CH(2)-NH" scan revealed that incorporation of reduced bonds at position 28-29 or 35-36 imparted greater receptor affinity. In general, disubstituted analogues designed based on the results of single substitutions exhibited goo receptor affinity with N-alpha-Ac-[Trp(27),CH(2)-NH(35-36)]PYY(22-36)-NH(2) having the greatest affinity (IC(50) = 0.28 nM). Conservative multipl substitutions with Nle-->Leu and Nva-->Val also imparted good affinity. An analogue designed to encompass most of the favored substitutions, N-alpha-Ac-[Nle(24,28),Trp(30),Nva(31), CH(2)-NH(35-36)]PYY(22-36)-NH(2), exhibited a proabsorptive effect in dogs comparable to, but longer lasting than, that of intact hormone. Selected analogues also exhibited good antisecretory potencies in rats with N-alpha-Ac-[Trp(30)]PYY(22-36)-NH(2) being even more potent than PYY. However, the potencies did not correlate well with the PYY receptor affinity or the proabsorptive potencies in dogs.

These differences could be due to species effects and/or the involvement of multiple receptors and neuronal elements in controlling the in vivo activity o PYY compounds. PYY(22-36) analogues exhibited good affinity for neurona Y2 receptors but poor affinity for Y1 receptors. Also, crucial analogues in this series hardly bound to Y4 and Y5 receptors. In summary, we have developed PYY(22-36) analogues which, via interacting with intestinal PYY receptors, promoted potent and long-lasting proabsorptive and antisecretory effects in in vivo models. These compounds or analogues based on them may have useful clinical application in treating malabsorptive disorders observed under a variety of conditions.

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# Synthetic Peptide YY Analog Binds to a Cell Membrane Receptor and Delivers Fluorescent Dye to Pancreatic Cancer Cells

*Carson D. Liu, M.D., David Kwan, Natalie Simon, David W. McFadden, M.D.*

Pancreatic cancer continues to have a dismal prognosis despite multimodality treatment plans. Peptide YY (PYY) is a gut hormone that suppresses pancreatic exocrine and endocrine function. Previous experiments have shown that shortened synthetic PYY(22-36) analog decreases pancreatic cancer cell growth while also decreasing intracellular cyclic adenosine monophosphate. Our purpose was to construct an optimal synthetic PYY analog that binds to pancreatic cancer cells that may be used for imaging and therapy. Biotinylated PYY analogs with lengths ranging from PYY(1-36), PYY(9-36), PYY(14-36), PYY(22-36), and PYY(27-36) were tested with flow cytometry and receptor cross-linking studies to measure cell membrane binding. Growth inhibition studies were also performed using monotetrazolium tests to determine potency of various PYY analogs. Quantitative flow cytometry reveals the highest specific binding of PYY(14-36) to pancreatic cancer cells. Cross-linking studies reveal a receptor on the cell membrane of human pancreatic ductal adenocarcinoma cells. Growth inhibition studies reveal that PYY (14-36) has the highest potency against PANC-1 and MiaPaCa-2 cells. A novel synthetic PYY analog binds to the cell surface of pancreatic cancer cells and has the ability to deliver fluorescent dyes. The strategy of using biotinylated peptides to deliver avidin-dye complexes to cancer cells will allow imaging of pancreatic tumors and delivery of therapeutic agents. (J GASTROINTEST SURG 2001;5:147-152.)

KEY WORDS: Peptide YY, pancreatic cancer, biotin

Pancreatic adenocarcinoma remains one of the most devastating neoplasms of the gastrointestinal tract. Pancreatic cancer is a malignancy that is unresponsive to conventional therapy. More than 85% of patients have metastatic disease when they are first seen. The incidence of pancreatic cancer is 9 per 100,000[1] and has remained steady since 1973.[2] Median survival on diagnosis is 11 months, whereas adjuvant treatment (5-fluorouracil and radiation treatment) with surgical resection (Whipple procedure) has extended life by approximately 9 months.[3] A dismal prognosis is associated with pancreatic adenocarcinoma despite multimodality treatment protocols. Although total pancreatectomy in selected patients offers survival advantages in rare cases, the difference remains negligible.[4] Earlier diagnosis and novel treatment modalities may help to improve survival in patients with pancreatic cancer.

The production of growth factors along with their receptors is a crucial step in triggering growth response in tumor cells.[5] Endogenous gastrointestinal hormones given at pharmacologic doses may provide another mode of adjuvant treatment. Previous works describe the growth-inhibiting properties of the synthetic peptide YY (PYY) analog PYY(22-36) on human pancreatic ductal adenocarcinomas in vitro[6] and

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Exhibit 2

in vivo.[7] A 2.5-fold difference in human pancreatic cancer mass is seen in nude mice exposed to PYY (22-36) for 2 weeks.[7] Furthermore, a noticeable decrease in the second messenger, cyclic adenosine monophosphate, is observed with the addition of synthetic PYY analog,[7] suggesting alteration of cellular metabolism. Synthetic PYY analogs augment the effects of 5-fluorouracil and leucovorin.[8] A decrease in cell membrane epidermal growth factor receptor protein expression is observed after treatment with PYY analogs and chemotherapy.[8]

We hypothesized that various lengths of PYY analogs would optimally bind to pancreatic cancer cells. Native PYY(1-36), PYY(9-36), PYY(14-36); PYY(22-36), and PYY(27-36) were constructed. These fragments were studied because of three-dimensional crystallographic structure data. Peptide YY has a polyproline type II–like alpha helix in residues 1 to 8, a beta turn in residues 9 to 13, and an alpha helix in residues 14 to 32 with a flexible tail in residues 33 to 36.[9] Biotin was covalently linked to the nonbinding side of the peptide, the amino terminal. The specific binding of biotinylated synthetic PYY to cancer cells would allow delivery of fluorescent dyes to image and possibly treat pancreatic cancer.

## MATERIAL AND METHODS

### In Vitro Growth of Human Pancreatic Cancer Cells

Human pancreatic ductal adenocarcinoma cell lines MiaPaCa-2 and PANC-1 (American Type Culture Collection, Rockville, Md.) were purchased and grown in Costar T125 flasks (Corning, Inc., Corning, N.Y.). Cells were grown in monolayers in RPMI 1640 medium supplemented with 10% fetal calf serum, 5 ml of 29.2 mg/ml L-glutamine (Irvine Scientific, Santa Ana, Calif.), 25 μg of gentamicin, and 5 ml of penicillin, streptomycin, and fungizone solution (JRH Biosciences, Lenexa, Kan.) at 37° C in a Forma Scientific (Marietta, Ohio) water-jacketed 5% carbon dioxide incubator. All cell lines were detached with 0.25% trypsin (Clonetics, San Diego, Calif.) once or twice a week. Cells were washed by centrifugation at 4° C at 500 *g* for 7 minutes. Viable cells were counted by trypan blue exclusion on a hemocytometer slide.

### Monotetrazolium Growth Assays of Biotinylated PYY Analogs

Biotinylated synthetic PYY analogs were constructed by Peninsula Laboratories (Belmont, Calif.) and added to MiaPaCa-2 and PANC-1 human pancreatic adenocarcinoma cells. A total of 30,000 cells were exposed to various concentrations of biotinylated PYY(14-36), bio-PYY(22-36), bio-PYY(27-36), and bio-(9-36) for 12 hours. Concentrations of peptides ranged from 10 nmol/L to 10 pmol/L in a volume of 200 μl with serum-free fortified RPMI 1640. After peptide treatment, monotetrazolium (MTT) assay was performed by the addition of 3-(4,5-dimethylthiazol-2-yl)-2, 5-dephenyltetrazolium bromide (Sigma, St. Louis, Mo.) at a final concentration of 0.5 mg/ml. Cells were incubated with tetrazolium for 3 hours prior to cessation of reaction. Formazon crystals were dissolved with 200 μl of dimethylsulfoxide. MTT assays were read on a Bio-Rad enzyme-linked immunosorbent assay microplate reader (Bio-Rad Laboratories, Inc., Hercules, Calif.) at 550 nm. The MTT assay measures mitochondrial NADH-dependent dehydrogenase activity, and it has been among the most sensitive and reliable methods for quantitating in vitro chemotherapy responses in tumor cells.[10]

### Quantitative Flow Cytometry

MiaPaCa-2 and PANC-1 cells ($1 \times 10^7$) were incubated at 4° C with biotinylated PYY analogs (10 nmol/L, 100 pmol/L) for 30 minutes in cold buffer at 4° C (0.2% albumin and 0.1% sodium azide). Cells were washed twice with phosphate-buffered saline (PBS), spun at 250 *g* for 5 minutes, and incubated with 1.67 μl of phycoerythrin-streptavidin (Caltech Labs, Burlingame, Calif.; stock solution = 150 μg/0.5 ml) in cold buffer for an additional one-half hour. Excess phycoerythrin was washed off twice with PBS and resuspended. Sample tubes were maintained in the dark with aluminum foil wrapping at 4° C and immediately submitted for flow cytometry within 30 minutes.

Flow cytometry was performed at core facilities located at our institution. Quantitative flow cytometry was programmed for phycoerythrin and raw counts were used to determine relative increases in fluorescent detection with escalating doses of biotinylated PYY analogs while maintaining the same concentration of phycoerythrin-streptavidin.

### Receptor Cross-Linking Studies

MiaPaCa-2, PANC-1, AR42J, and 3T3 cells were trypsinized and washed with RPMI 1640 serum-free medium. Cells were resuspended at $1.0 \times 10^6$ cells/ml and added to synthetic biotinylated PYY analogs at 10 nmol/L per reaction vial for 1 hour at 4° C on a shaker. Cells were resuspended after washing with PBS, pH 8.0, and 1 mmol/L magnesium chloride at 4° C. Water-soluble cross-linking agent, BS3 (Pierce, Rockford, Ill.), was added to cells to form a final concentration of 20 μg/ml. Cells were tumbled with

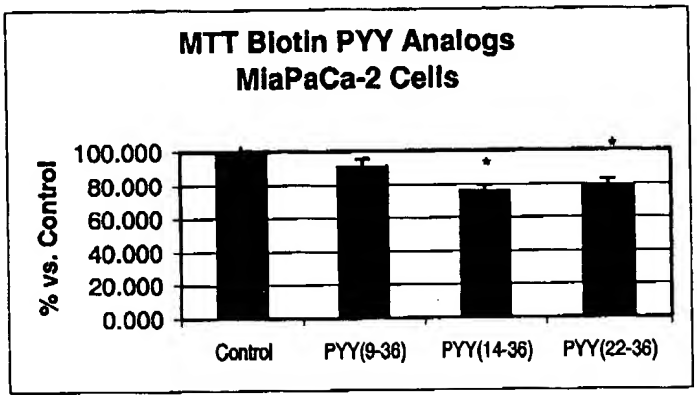


Fig. 1. Growth inhibition of MiaPaCa-2 pancreatic cancer cells exposed to various lengths of biotinylated PYY analogs.

cross-linking buffer for 20 minutes on a rocking platform. One volume of TE buffer (Tris, EDTA, pH 7.4) was added to stop the reaction. Cells were collected with centrifugation at 4° C. Pooled cells were subjected to cell lysis buffer (300 mmol/L NaCl, 50 mmol/L Tris-Cl, pH 7.6, 0.5% Triton X-100 with protease inhibitors, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mmol/L phenylmethylsufonyl fluoride, and 1.8 mg/ml iodoacetamide).

Solubilized cell membranes were separated on a 4% to 12% gradient gel (Bio-Rad Laboratories). Concentrated solubilized cell membranes were added to Laemmli's reducing sample buffer in a ratio of 1:4 (1 ml of 0.5 mol/L Tris-HCl, pH 6.8, 800 μl of glycerol, 1.6 ml of 10% sodium dodecyl sulfate, 400 μl of 2-b-mercaptoethanol, 200 μl of 0.05% bromophenol blue, and 4 ml of distilled water). Electrophoretic separation was performed at 80 volts for 3 hours. Biotinylated molecular weight marker (Pierce) was simultaneously loaded in a different lane to identify the molecular weight of the receptor. Gels were removed from the plastic plates and protein transfer to nylon membrane (Stratagene, Austin, Tex.) was performed overnight at 30 volts. Nylon membranes were probed with streptavidin–horseradish peroxidase provided by ECL kits (Amersham, Buckinghamshire, England). Blots were exposed to Hyperfilm after addition of developing reagents (Amersham).

## RESULTS

### Growth Inhibition Studies of Biotinylated Peptides

Growth of both MiaPaCa-2 and PANC-1 cell lines were maximally inhibited by biotinylated PYY(14-36) at 10 nmol/L. Biotinylated PYY(27-36) did not have any biologic activity against pancreatic ductal adenocarcinoma cell growth. MiaPaCa-2 cell growth inhibition is depicted in Fig. 1 with the addition of 10 nmol/L bio-PYY(14-36) after a 12-hour exposure time. Growth inhibition is described as the percentage of reduction versus control groups. Control groups received an equivalent amount of PBS (peptide solvent) to mimic the physical disturbance of adding peptide solution in other wells ($N = 12$; $P < 0.05$ by analysis of variance). Biotinylated PYY (14-36) reduces the growth of MiaPaCa-2 by 24% ± 1.2% and PANC-1 cells by 30% ± 1.8% after a 12-hour exposure. MTT studies were performed 72 hours after initiation of exposure to peptide therapy. These studies reveal that biologic activity is maintained with a biotin group covalently linked to the amino terminal. Biotin-PYY(14-36) is the most effective synthetic analog in suppressing pancreatic cancer growth in both cell lines.

### Flow Cytometry of Biotinylated Peptide YY With Streptavidin Phycoerythrin

Cell surface binding of human pancreatic cancer cell lines MiaPaCa-2 and PANC-1 was studied in our laboratory by quantitative flow cytometry. Various lengths of biotinylated PYY analogs were studied for their ability to deliver a fluorescent dye, phycoerythrin. Attached to the phycoerythrin is streptavidin. The streptavidin has a high affinity to the biotin group on the PYY analogs. PYY(9-36) did not have significant binding of flow cytometry. PYY(14-36) was effective at lower concentrations than PYY(22-36).

Fig. 2 depicts two different pancreatic ductal adenocarcinoma lines, MiaPaCa-2 and PANC-1, with specific binding up to 47% ± 3% at the higher concentration of 10 nmol PYY(14-36) per 500,000 cancer cells. Quantitative flow cytometry was performed with streptavidin-phycoerythrin as the fluorescent conjugate to biotinylated PYY(14-36). Data are pre-

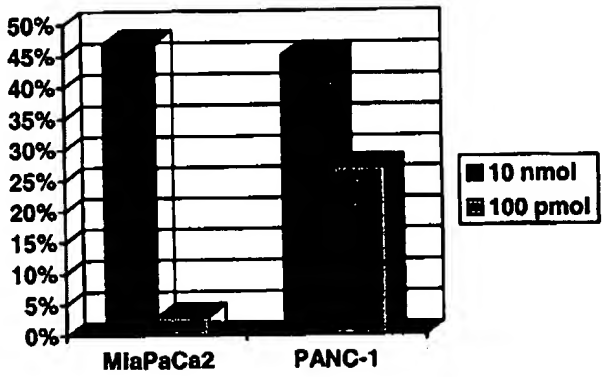


Fig. 2. Percentage of receptor binding of biotinylated PYY-(14-36) as estimated by raw counts obtained by quantitative flow cytometry. PYY(14-36) had the greatest amount of cell membrane binding as detected by an indirect method that uses streptavidin-phycoerythrin to detect the biotinyl group located on the amino terminus of PYY(14-36). No binding is observed in 3T3 fibroblasts, and minimal binding is observed in AR42J cell lines.

sented as the percentage with phycoerythrin fluorescent binding beyond background binding where phycoerythrin-avidin was added without peptide analogs ($N = 6$; $P < 0.05$ by analysis of variance). Histogram shifts are observed when biotinylated PYY(14-36) is added at increasing concentrations. Cancer cells were washed twice with PBS prior to flow cytometry to remove any unbound peptides and washed twice after the addition of streptavidin-phycoerythrin conjugate. Background binding was consistently below 2%. Quantitative flow cytometry proves that biotinylated PYY(14-36) has the ability to bind to the surface of pancreatic cells and allow streptavidin-phycoerythrin to bind to the peptide.

## Receptor Cross-Linking Studies

Cross-linking with BS3 (Pierce) was performed with biotinylated PYY(14-36) and pancreatic cancer cells. After incubation with BS3, reactions were stopped and cell membranes were harvested for gel electrophoresis. Standard protein gel separation was performed and revealed a consistent receptor band with biotinylated PYY(14-36) linked to a protein approximating 68 kdal. This band has been found consistently in pancreatic cancer cells and not in fibroblast cell lines when biotinylated PYY(14-36) is added during receptor cross-linking studies (Fig. 3).

Fig. 4 depicts MiaPaCa-2 adenocarcinoma cells pretreated with biotinylated PYY(14-36) and probed with streptavidin-phycoerythrin. Cells were diluted with 50% glycerol and examined with fluorescent microscopy. The photograph depicts fluorescent

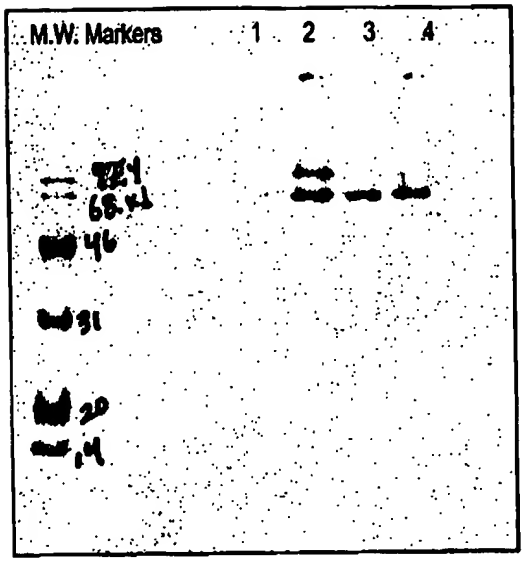


Fig. 3. Cross-linking studies using biotinylated PYY(14-36) and separating the solubilized cell membrane fragments over a protein separating gel. The first lane has biotinylated molecular weight markers. Lane 1 = 3T3 fibroblasts; lane 2 = AR42J pancreatic acinar cancer cells; lane 3 = MiaPaCa-2 ductal adenocarcinoma cells; lane 4 = PANC-1 ductal adenocarcinoma cells.

Fig. 4. Fluorescent microscopy of MiaPaCa-2 cells with biotinylated PYY(14-36) and streptavidin-phycoerythrin. Similar fluorescence is observed in PANC-1 cell lines and no fluorescence is observed with AR42J and 3T3 cell lines during microscopy.

cells after vigorous washing of excess streptavidin-phycoerythrin from cells.

## DISCUSSION

The initial finding that a shortened PYY analog had growth-suppressing effects was surprising. Previous receptor binding studies have revealed no binding of native PYY to cancer cells, but binding does exist with the shortened PYY(22-36) and PYY(14-36). We have described additional evidence and techniques proving that an improved analog of PYY, biotinylated PYY(14-36), has the ability to

bind to pancreatic cancer cells and deliver fluorescent dye attached to streptavidin. The implications of this streptavidin-biotin indirect model will allow delivery of other fluorescent agents that are more toxic when photoactivated, or other radioactive compounds that may be used for imaging or treatment against metastatic disease. Previous studies by our group have shown that a significant decrease is observed during in vivo administration of synthetic shortened PYY analogs when administered over 2 to 4 weeks.[7] Prolonged exposure to the synthetic PYY analogs will geometrically increase the growth suppression. Pancreatic cancer cells were exposed to peptides for 12 hours and assayed at 72 hours after exposure to peptides. These growth suppressions are remarkable after a short period of exposure to synthetic peptides.

The ability to isolate a novel receptor may be helpful in designing newer antibodies or receptor antagonists to decrease growth of pancreatic cancer. The single band on pancreatic ductal adenocarcinoma cell lines may represent a novel receptor on pancreatic cancer cells. Although verification of this receptor will have to be performed in freshly harvested pancreatic cancer tissue, the ability to localize this single band on tissue culture cell lines is encouraging. Of interest is the ability to isolate a second band on AR42J pancreatic acinar cell lines. The heavier protein band may represent a secondary binding site on acinar cell lines as compared to ductal adenocarcinoma cell lines. The difference may also be explained by the difference in species.

From our previous studies using PYY(22-36), we have seen dramatic decreases in pancreatic cancer cell growth and concomitant decreases in intracellular cyclic adenosine monophosphate.[7] Y receptors have been extensively studied since the sequencing of the first subclass in 1992, and five different Y subtypes have been described to date.[11] All Y-subtype receptors seem to maintain the seven transmembrane regions and their association with G proteins. It remains to be determined whether PYY(14-36) binds to a novel version of Y-subtype receptors or whether this is a new receptor altogether. It would be feasible that this receptor is novel and does not exist in the Y subtype because of our inability to prove binding of native PYY or NPY(3-36) in our previous work. These two peptides represent ligands for Y1- and Y2-subtype receptors, respectively.

The ability to isolate a new receptor may allow us to clone the receptor and use the expression of this receptor as a prognostic indicator for patients with pancreatic cancer. The quantitative expression of growth receptors has been studied in breast cancer with estrogen, epidermal growth factor, and Her-2-neu. We have not quantified the number of receptors with biotinylated PYY(14-36). In our previous work we did calculate 27,000 receptors per cell by Scatchard analysis.[7] Because PYY(14-36) has a higher affinity for pancreatic cancer cells than PYY(22-36), competitive receptor studies will have to be repeated with radioactive iodine-125. It is difficult to quantify the number of receptors by flow cytometry because variable numbers of ligands bind to each cell. Furthermore, the expression of this PYY(14-36) receptor may exist in other malignancies in addition to pancreatic cancer. The advantage of using a shortened synthetic analog is its toxicity is minimal because it is a shortened analog of an endogenous gut hormone, PYY, and its ability to uniquely bind to cancer cells. The shortened peptide is more specific than the previously described PYY(22-36), and it has the ability to deliver more ligands to the cell surface as observed in flow cytometry, receptor cross-linking studies, and fluorescent microscopy.

The ability to decrease pancreatic cancer cell growth and bind to a unique cell membrane protein may prove useful in imaging and treating pancreatic cancer. We have shown the ability to deliver a nontoxic agent, phycoerythrin, by using an indirect method of binding a synthetic analog of PYY to cancer cells followed by streptavidin-phycoerythrin. The extremely high affinity of streptavidin to biotin is useful in vitro, but further studies will have to be performed in vivo. Naturally occurring biotin may interfere with this model of dye delivery.

## CONCLUSION

Biotinylated PYY(14-36) decreases pancreatic cancer cell growth while also binding to a specific cell membrane receptor. The implication of discovering a new receptor on pancreatic cancer cells will be useful for future therapy and detection. If a specific receptor is solely expressed on pancreatic cancer cells, earlier detection of pancreatic cancer may be feasible. Eighty-five percent of patients have unresectable pancreatic cancer when initially seen. If more of these patients were diagnosed earlier, survival after surgical resection and chemotherapy might be improved.

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## *Discussion*

***Dr. S.B. Archer*** (Boston, Mass.). Why do you think the shortened version of PYY works so much better with this receptor versus the full length? Are you planning to do any in vivo studies to follow up on these findings?

***Dr. C. Liu.*** The full length must have a three-dimensional conformity that does not bind with cancer cells. I do not know whether or not this is the case only in tissue culture cells, but we do know that when just two amino acids are taken off the end tumors of PYY, it changes the entire fold of the peptide. It starts to unfold. We are in the process of using a mouse model in which we orthotopically grow human cancer in the pancreas as well as in the liver and see if we can image the cells.

1: J Gastrointest Surg. 2001 Mar-Apr;5(2):147-52. Related Articles, Link

ELSEVIER FULL-TEXT ARTICLE

# Synthetic peptide YY analog binds to a cell membrane receptor and delivers fluorescent dye to pancreatic cancer cells.

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Pancreatic cancer continues to have a dismal prognosis despite multimodality treatment plans. Peptide YY (PYY) is a gut hormone that suppresses pancreatic exocrine and endocrine function. Previous experiments have shown that shortened synthetic PYY(22-36) analog decreases pancreatic cancer cell growth while also decreasing intracellular cyclic adenosine monophosphate. Our purpose was to construct an optimal synthetic PYY analog that binds to pancreatic cancer cells that may be used for imaging and therapy. Biotinylated PYY analogs with lengths ranging from PYY(1-36), PYY(9-36), PYY(14-36), PYY(22-36), and PYY(27-36) were tested with flow cytometry and receptor cross-linking studies to measure cell membrane binding. Growth inhibition studies were also performed using monotetrazolium tests to determine potency of various PYY analogs. Quantitative flow cytometry reveals the highest specific binding of PYY(14-36) to pancreatic cancer cells. Cross-linking studies reveal a receptor on the cell membrane of human pancreatic ductal adenocarcinoma cells. Growth inhibition studies reveal that PYY (14-36) has the highest potency against PANC-1 and MiaPaCa-2 cells. A novel synthetic PYY analog binds to the cell surface of pancreatic cancer cells and has the ability to deliver fluorescer dyes. The strategy of using biotinylated peptides to deliver avidin-dye complexes to cancer cells will allow imaging of pancreatic tumors and delivery of therapeutic agents.

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# Acute effects of $PYY_{3-36}$ on food intake and hypothalamic neuropeptide expression in the mouse

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**Abstract**

It has recently been suggested that gut-derived $PYY_{3-36}$ may be involved in the central mediation of post-prandial satiety signals. We have examined the acute effects of peripherally administered $PYY_{3-36}$ on food intake and hypothalamic gene expression of neuropeptides in mice. A single intraperitoneal injection of $PYY_{3-36}$ to mice that had been fasted for 24 h resulted in a highly significant reduction in food intake at 6 and 24 h post-injection but not at 48 h. However, in freely fed mice, food intake was unaltered by $PYY_{3-36}$ administration. In the arcuate nucleus POMC mRNA expression was significantly elevated at 6 h and remained elevated at 24 h following $PYY_{3-36}$ injection. By contrast NPY mRNA expression in the arcuate nucleus was suppressed at 6 h but not at 24 h post-injection. In the lateral hypothalamus there were no differences in MCH mRNA expression at either time point. In conclusion, peripherally administered $PYY_{3-36}$ has a suppressive effect on food intake that is more prominent in recently fasted mice and lasts up to 24 h. This is associated with a short-lived suppression of NPY mRNA, a longer lasting increase in POMC mRNA but no change in MCH mRNA expression.


Neurons within the hypothalamic arcuate nucleus maintain energy homeostasis through the integration of a number of peripheral and central signals related to energy status [1–3]. Peptide-$YY_{3-36}$ ($PYY_{3-36}$), a member of the neuropeptide Y (NPY) family that is secreted from endocrine L cells of the gastrointestinal tract in response to caloric ingestion, has recently been suggested to be a possible mediator of post-prandial satiety in rodents [3] and human [4] through actions at the level of the arcuate nucleus. The peripheral administration of $PYY_{3-36}$ has recently been described to potently suppress food intake in rodents and to induce the expression of *c-fos* in POMC-expressing neurons of the arcuate nucleus acting through the Y2-receptor (Y2-R). The Y2-R is a pre-synaptic inhibitory receptor that is abundantly expressed on NPY neurons in the arcuate nucleus [5] and $Y2R^{-/-}$ mice have been shown to be resistant to the anorectic effects of $PYY_{3-36}$ [3]. Electrophysiological studies indicated that $PYY_{3-36}$ was inhibitory to NPY neurons and blocked the inhibitory effects of NPY neurons on adjacent POMC neuronal activity [3]. Recent studies from other workers have, however, challenged this concept [6] and it is therefore critical to further test this new and potentially therapeutically relevant model of post-prandial satiety. We have therefore examined the acute effects of peripherally administered $PYY_{3-36}$ on food intake and hypothalamic neuropeptide gene expression in mice.

## Materials and methods

*Animals.* Two-month-old wild-type 129/J male mice were used for all experiments. Mice were housed in a temperature-controlled room (25–27 °C) under a 12-h light (7:00–19:00)/12-h dark cycle, given free access to water, and fed ad libitum on a standard chow unless otherwise stated. All procedures were done in accordance with UK Home Office guidelines for animal care.

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Exhibit 3

*Evaluation of food intake.* For measurement of food consumption mice were individually housed in cages two weeks before the beginning of the experiment. Mice with ad libitum access to food or fasted for 24 h were intraperitoneally injected with 100 µl $PYY_{3-36}$ (Bachem, UK) (10 µg/100 g of body weight) or saline at the onset of the dark cycle (19:00) and food intake was measured at 6, 24, and 48 h following compound administration from pre-weighed portions of food dispensed from wire cage tops. Cages were carefully monitored for evidence of food spillage or grinding, which was negligible.

*In situ hybridisation.* Freely feeding mice were injected with 100 µl $PYY_{3-36}$ (10 µg/100 g of body weight) or saline and sacrificed following 6 or 24 h. Brains were dissected and immediately frozen on dry ice. Ten micron brain sections were fixed with 4% paraformaldehyde (Sigma, Dorset, UK) for 5 min, washed in PBS, and then dehydrated in 70% ethanol and 95% ethanol for 5 min each. Antisense oligonucleotides probes complementary to the mRNA of the neuropeptides investigated were labelled with $^{35}S$. Two microlitres of purified oligonucleotide (5 ng/µl) was added to 2.5 µl buffer (Roche Diagnostic, Sussex, UK). DEPC-treated water (5.25 µl) was added, followed by 1 µl $^{35}S$-deoxyadenosine 5-(α-thio)-triphosphate (10 nCi/ml) (Amersham Biosciences, UK), 1.25 µl $CoCl_2$, and 0.5 µl (15–20 U) of terminal deoxynucleotide transferase enzyme (Roche Diagnostic). The reaction was incubated at 37 °C for 1 h and terminated by addition of 40 µl DEPC water. Purification of the labelled probe from unincorporated nucleotides was accomplished by centrifugation through a ProbeQuant G50 Microspin Column (Amersham Biosciences). Probes were then evaluated for incorporation of radiolabel by scintillation counting. All hybridisations were carried out at 5000 cpm/µl in hybridisation buffer (50% deionised formamide, 20× SSC, 5× Denhardt's, 5 mg/ml polyadenylic (potassium salt) acid, 10 mg/ml salmon sperm DNA, 120 mg/ml heparin (BDH, Leicestershire. UK), and 0.5 M sodium phosphate; pH 7, 0.1 M sodium pyrophosphate, 10% (w/v) dextran sulphate in DEPC-treated water (Sigma, Dorset, UK)). After incubation at 42 °C overnight with a labelled probe, slides were washed and air-dried at room temperature before exposure to X-ray film (Amersham Biosciences). The duration of exposure to the X-ray film varied according to the mRNA transcript under investigation.

*Analysis and quantification.* For quantification of POMC and NPY mRNA in the arcuate nucleus and MCH mRNA in the lateral hypothalamic area, the exposure time to the autoradiographic film was 5–7 days. The sections were analysed and compared against a $^{14}C$-labelled standard of known radioactivity (Amersham, UK). The optical density of the autoradiographic images was measured by using a computerised Macintosh-base image analysis system (NIH Image). Optical densities were obtained from six consecutive sections per mouse and the average value for each animal was used to calculate group means.

## Results

### *Food intake*

Food intake was measured in $PYY_{3-36}$ and vehicle-treated mice in response to peripherally administered $PYY_{3-36}$ (10 µg/100 g body weight) at 6, 24, and 48 h post-injection. In freely feeding mice, a trend towards reduced food intake was observed in $PYY_{3-36}$-treated mice at 6 and 24 h post-injection, however this did not reach statistical significance (Fig. 1A). In fasted mice treated with $PYY_{3-36}$, food intake was significantly inhibited at 6 and 24 h post-injection (Fig. 1B). By 48 h, food intake in $PYY_{3-36}$-treated mice was similar to saline controls in both fed and fasted animals. There was no significant difference in body weight between $PYY_{3-36}$ and vehicle-treated mice at any time point (data not shown).

### *POMC, NPY, and MCH mRNA expression*

To investigate the impact of $PYY_{3-36}$ administration on orexigenic and anorexigenic pathways in the hypothalamus, in situ hybridisation was used to determine hypothalamic NPY, POMC, and MCH mRNA expression in freely feeding mice treated with $PYY_{3-36}$ and vehicle at 6 and 24 h following injection. These time points were chosen based on a preliminary study in which the time course of POMC mRNA expression was determined at 2, 4, 6, and 24 h and at 1 week following injection of $PYY_{3-36}$ and shown to be most prominent at 6 h and 24 h (data not shown). In the arcuate nucleus, POMC expression was elevated by approximately 6- and 2.4-fold at 6 and 24 h, respectively, in $PYY_{3-36}$ injected animals compared with vehicle controls (Figs. 2A and 3). Furthermore, $PYY_{3-36}$ treatment suppressed NPY mRNA in the arcuate nucleus by 2.3-fold at 6 h (Fig. 3) but by 24 h the mRNA levels increased to match expression levels of saline

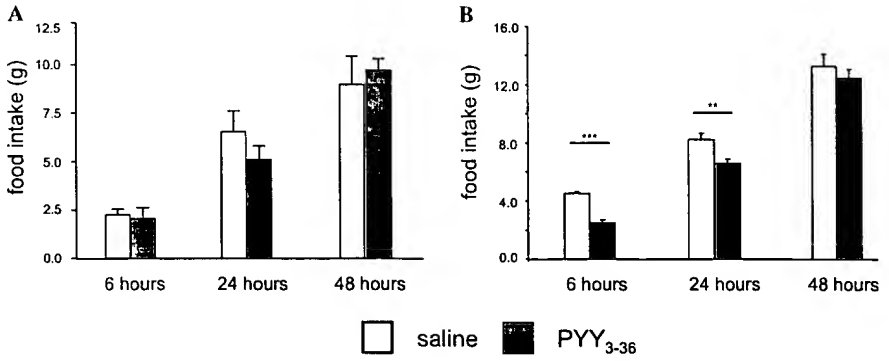


Fig. 1. Feeding response to a single i.p. injection of $PYY_{3-36}$ (10 µg/100 g body weight) or saline in (A) freely feeding and (B) 24 h fasted mice. Data are means ± SEM ($n = 8$ per group); ***, $P < 0.001$; **, and $P < 0.01$ versus saline, one-way ANOVA.

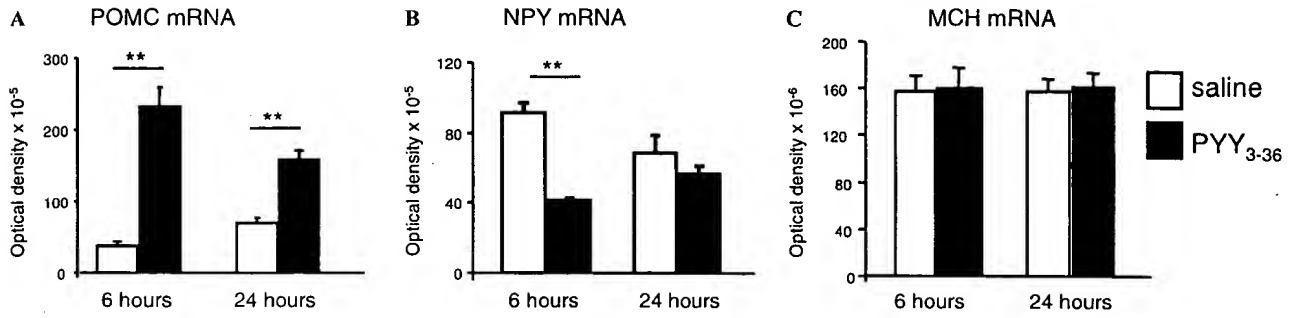


Fig. 2. Hypothalamic mRNA expression of POMC, NPY, and MCH in freely feeding mice treated with a single i.p. injection of $PYY_{3-36}$ (10 μg/100 g body weight) or saline as determined by in situ hybridisation. In the arcuate nucleus, POMC expression was elevated by approximately 6- and 2.4-fold at 6 and 24 h, respectively, in $PYY_{3-36}$- treated animals compared with vehicle (all $P < 0.01$, one-way ANOVA). $PYY_{3-36}$ treatment suppressed arcuate NPY expression 2.3-fold at 6 h ($P < 0.01$) but had no effect on expression levels 24 h post-injection. No differences in MCH mRNA expression in the lateral hypothalamus were observed between $PYY_{3-36}$ and saline-treated mice at either time point. Results are expressed as means ± SEM optical density (pixels) for each experimental group ($n = 5$ per group).

controls (Fig. 2B). Analysis of MCH mRNA expression in the lateral hypothalamus revealed no differences between $PYY_{3-36}$ and saline-treated mice at either time point (Fig. 2C).

## Discussion

The results presented here are in agreement with those published by Batterham et al. and demonstrate

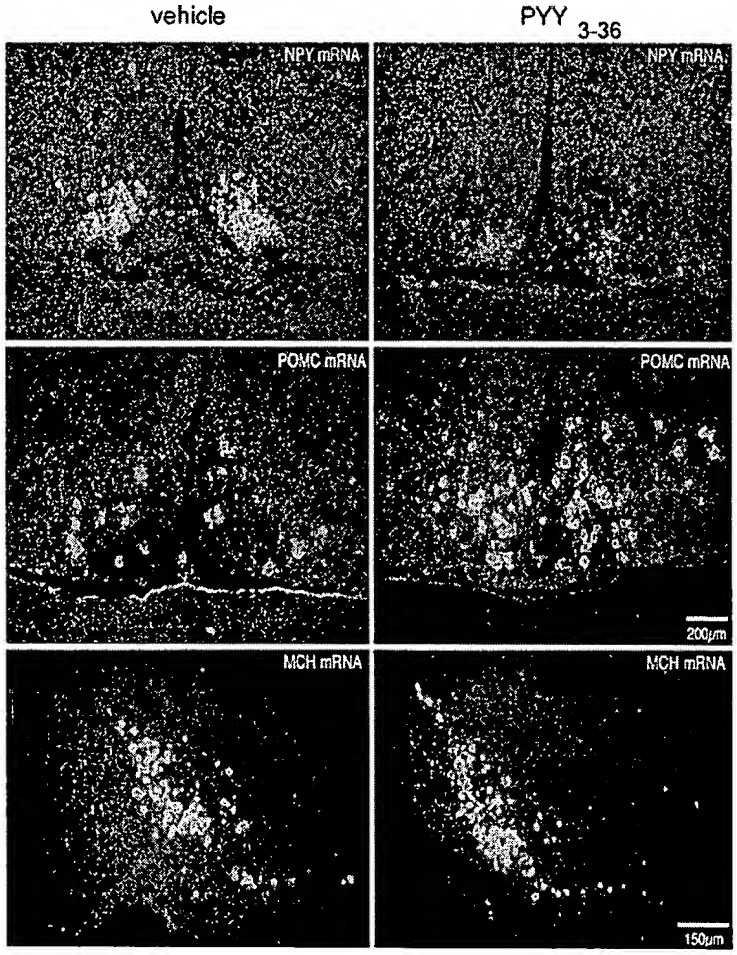


Fig. 3. Representative dark-field photomicrographs of coronal hypothalamic sections from freely feeding mice treated with $PYY_{3-36}$ (10 μg/100 g body weight) or saline, sacrificed 6 h post-injection, and analysed for POMC, and NPY mRNA in the arcuate nucleus and MCH mRNA expression in the lateral hypothalamus by in situ hybridisation. The same scale is used for the POMC and NPY photomicrographs.

that peripherally administered $PYY_{3-36}$ has acute, suppressive effects on food intake that appear to be mediated, at least in part, by the hypothalamus. We found that $PYY_{3-36}$ reduced food intake, an effect that appears to be exaggerated in the fasted state and observed up to 24 h post-injection. Furthermore, these effects were associated with reciprocal changes in the level of hypothalamic mRNA expression of NPY and POMC that are consistent with the known roles of these neuropeptides in feeding.

$PYY_{3-36}$ is expressed at increasing levels along the gastrointestinal tract with the highest concentrations found in the terminal ileum, colon, and rectum [7]. $PYY_{3-36}$ is initially released from endocrine L cells shortly following food ingestion likely via a neural mechanism whereas post-prandial release occurs in response to carbohydrates and fats within the distal lumen of the small bowel and colon. Although studies to date suggest that the hypothalamus is the target through which $PYY_{3-36}$ reduces food intake, additional effects through the afferent vagus or brainstem have not been excluded. Indeed, it has been demonstrated that $PYY_{3-36}$ inhibits excitatory synaptic transmission in the brainstem [8], however, Batterham et al. [3] have shown that $PYY_{3-36}$ does not appear to affect gastric emptying. If $PYY_{3-36}$ does act through the hypothalamus it might be expected to affect the expression of POMC and/or NPY gene expression in the arcuate nucleus. Both neuroanatomical [5] and electrophysiological [3] data support a mechanism in which $PYY_{3-36}$ is inhibitory to NPY neurons thereby removing GABA mediated tonic inhibitory effects on adjacent POMC neurons. Using in situ hybridisation we found that POMC expression was dramatically increased and sustained for 24 h in freely feeding mice treated with $PYY_{3-36}$. In contrast, Batterham et al. found non-significant increases in POMC mRNA levels, which may reflect a difference in the techniques employed to evaluate gene expression. In agreement with Batterham et al., we found that NPY mRNA levels were suppressed at 6 h following $PYY_{3-36}$ treatment. However, this was not a sustained effect and by 24 h NPY mRNA levels reached those observed for saline-treated controls.

Despite our data, however, the effects of $PYY_{3-36}$ on POMC and NPY mRNA expression did not clearly correlate with major effects on food intake in freely feeding mice. Thus, in the non-fasted state alternative pathways, either central or peripheral, may exist to promote food intake thereby overriding $PYY_{3-36}$ effects on appetite. Nevertheless, taken together our data suggest that POMC neural activity has a more prominent role than NPY inhibition in $PYY_{3-36}$-induced hypophagia.

Melanin-concentrating hormone (MCH) is a potent orexigenic peptide that is expressed in the lateral hypothalamus downstream of POMC neurons [9]. Furthermore, the *MCH* gene is upregulated in $A^y/a$ mice and rats treated with MC3/4-R antagonists [10], suggesting that MCH expression is negatively regulated by melanocortin peptides. Given the dramatic effects of $PYY_{3-36}$ on POMC mRNA levels, we were surprised not to observe any changes in MCH mRNA levels following $PYY_{3-36}$ administration. These data provocatively suggest that alternative neural pathways downstream of POMC neurons might mediate the anorexigenic actions of $PYY_{3-36}$. Such possible candidates include neurons expressing TRH or CRH, however, at present their precise chemical identity remains unclear.

In summary, these studies provide some support for the notion that $PYY_{3-36}$, delivered from the systemic circulation might have the capacity to act as a post-prandial satiety hormone, having its anorectic effects through modulation of POMC and NPY expression in the hypothalamus. Thus, $PYY_{3-36}$ or highly selective Y2-R agonists may offer an alternative therapeutic strategy for the treatment of human obesity.

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